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PHENOTYPE AND GENOTYPIC CHARACTERIZATION OF ESCHERICHIA COLI ISOLATED FROM NEONATAL CALVES WITH DIARRHEA

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FINAL REPORT (ADF PROJECT 20020027)

Phenotypic and genotypic characterization of *Escherichia coli* isolated from neonatal calves with diarrhea.

(a) ABSTRACT

To evaluate the potential of emerging enteropathogen, 408 and 635 *E. coli* isolates from feces of newborn to 2-week-old diarrheic and non-diarrheic calves, respectively, were tested for attachment factors F4, F5, F6, F17 and F41 fimbriae, for *E. coli* attaching and effacing (EAE), porcine attaching and effacing associated (Paa), and adhesin involved in diffuse adherence (AIDA-I). In addition, the isolates were tested for LT, STa, STb and EAST1 enterotoxins, for Shiga toxins (Stx1/Stx2), cytotoxic necrotizing factos (CNF1/CNF2) and for cytolethal distending toxins (CDT-3/CDT-4), using DNA hybridization. The isolates from the main pathotypes were tested for antibiotic sensitivity to 12 drugs and some of these isolates were O-serotyped. The majorities (78.4%) of isolates from diarrheic calves were assigned a pathotype, and 44.1% were positive for F17 fimbriae. The main pathotypes included EAST1 (24.5%), F17 (20.1), F17/EAST1 (14.5%), F17/CDT-3/CNF associated or not with (±) EAST1 (5.4%), F5/STa/Paa ± F17, F41 or EAST1 (4.4%) and EAE/Paa/EAST1 ± Stx1 (2.5%). Among isolates from non-diarrheic animals, 67.5% strains were assigned a pathotype, 34.3% were F17-positive and the main pathotypes included EAST1 (15.1%), F17 (14.2%), F17/EAST1 (6.3%), F17/CDT-3/CNF ± EAST1 (10.5%), EAE/Paa/EAST1 ± CDT-3, CNF, Stx1 or Stx2 (4.2%). No particular pattern was identified in the different O-serogroups. The antibiotic sensitivity profile revealed that isolates of pathotypes from diarrheic calves were frequently more resistant to antimicrobial drugs than isolates from non-diarrheic animals. Overall, our data show that F17/EAST1 and F17/CDT-3/CNF ± EAST1 may be considered significant *E. coli* pathotypes with potential role in neonatal calves'diarrhea. However, it is necessary to determine such role in experimentally inoculated newborn calves.

(b) EXECUTIVE SUMMARY

Enteric colibacillosis, also referred to as white scours, is an important cause of morbidity and mortality in livestock in general and represents the very common clinical syndrome of neonatal calf diarrhea. The detection of *E coli* enterotoxins in the early 1980's provided evidence that Enterotoxigenic *E. coli* (ETEC) plays an etiological key role in the disease. However, other pathogenic group of *E. coli*, such as shiga toxin-producing *E. coli*, has been isolated from cases of neonatal calf diarrhea, suggesting that ETEC should not be regarded as the only potential pathogenic group of *E coli* capable of producing the syndrome in calves.

Over the last few years, we have observed an increasing number of undiagnosed, but suspected cases of *E. coli* diarrhea in calves. Therefore, the objectives of the present study were to investigate the potential existence of emerging *E. coli* pathogens associated with enteric disease in newborn calves and to assess the antimicrobial profile of potential pathogenic *E. coli* groups to 12 drugs.

Our results show that a high number of *E. coli* strains from both diarrheic (78.4%) and non-diarrheic calves (67.5%) could be assigned a pathotype and that 44.1% and 34.3% of these isolates, respectively, were positive for F17 fimbriae. The main pathotypes recovered from diarrheic calves included EAST1 (24.5%), F17 (20.1%), F17/EAST1 (14.5%), F17/CDT-3/CNF associated or not with (±) EAST1 (5.4%), F5/STa/Paa ± F17, F41 or EAST1 (4.4%) and EAE/Paa/EAST1 ± Stx1 (2.5%) (Table 1 attached). Among isolates from non-diarrheic animals, the main pathotypes included EAST1 (15.1%), F17 (14.2%), F17/EAST1 (6.3%), F17/CDT-3/CNF ± EAST1 (10.5%), EAE/Paa/EAST1 ± CDT-3, CNF, Stx1 or Stx2 (4.2%) (Table 1). Minor pathotypes representing less than 1% of isolates each included F17/CDT-3, F17/CNF, CDT-3/CNF, AIDA-I ± EAST1, AIDA-I/CDT-3 and a variety of combinations between F17, STa, CDT-3, CDT-4, EAE, Paa, Stx1 and Stx2 factors, in both diarrheic and non-diarrheic animals. Among 50 selected isolates used for O-typing, 50% were untypable and various serogroups such as O26 and O8/O60 were observed in the F17/CDT-3/CNF and EAE pathotype. Thus, no particular pattern was identified in the different O-serogroups (results not shown). Antibiotic sensitivity test on isolates of the main pathotypes showed that strains from diarrheic calves were more frequently resistant to the drugs tested for than strains from non-diarrheic animals (see Table 2, attached). This suggests that usage of antimicrobial drugs in diarrheic calves probably lead to selection of highly resistance strains. On the other hand, all the strains

from both groups of calves were resistant to erythromycin, penicillin/novobiocin and tilmicosin, and approximately 50 to 90% of isolates were resistant to tetracycline (results not shown), suggesting that *E. coli* has probably adapted to these antimicrobial agents.

Overall, we have demonstrated that a high number of *E. coli* isolates from calves in Western Canada (Saskatchewan, Manitoba, Alberta and British Columbia) possess new combinations of virulence-associated factors, which are expressed simultaneously in the F17/EAST1 and F17/CDT-3/CNF (± EAST1) pathotypes. Although these pathotypes were found in both diarrheic and non-diarrheic calves, it is possible that the non-diarrheic calves shedding them are asymptomatic carriers and may develop diarrhea only under certain circumstances. Recently, we have demonstrated a similar situation in which an *E. coli* attaching and effacing (EAE) pathotype isolated from non-diarrheic pigs induced diarrhea when inoculated in newborn pigs (Ngeleka et al. 2003, J. Vet. Diagn. Invest. 15:242-252, ADF funded). Based on such observations, we believe that although *E. coli* F17/EAST1 and F17/CDT-3/CNF pathotypes were isolated in high numbers from both diarrheic and non-diarrheic calves, it is necessary to determine their role, if any, in neonatal diarrhea of calves.

It appears that despite the implementation of good vaccination program against neonatal calf diarrhea using ETEC anti fimbrial-based vaccines, calves infected with the main pathotypes described above may not be protected. Antibiotic therapy may cure the infection in a certain number of cases but as observed in the present study, development of resistance, especially in the diarrheic animals seems to lead to the emergence of highly resistant bacterial strains, making this practice inefficient.

(c) TECHNICAL REPORT

Project activities: The project account was set up in October 2002. The project started in November 1, 2003. By December 1, 2002 the DNA probes were designed and the first specimens and derived *E. coli* isolates were analyzed. As of May 30, 2003, DNA hybridization analysis, antibiotic sensitivity of isolates of major pathotypes and O-serotyping of selected isolates were completed.

NOTE: We had initially planned testing a total of 300 *E. coli* isolates from both diarrheic and non-diarrheic calves. We were fortunate to increase this number and had a chance to test 408 and 635 isolates from diarrheic and non-diarrheic calves, respectively. This was due to the

collaboration of Dr. Sheryl Waldner (Large Animal Clinical Sciences, WCVM) who is conducting a research project on "Prevalence of *E. coli* O157:H7 in asymptomatic cow-calf herds". She provided the isolates from non-diarrheic (asymptomatic) and some (approximately 50 isolates) from the diarrheic calves, used in our study. Dr. John Fairbrother (Department of veterinary microbiology, Faculty of veterinary medicine, University of Montreal) is highly interested in our project and has also provided assistance in DNA testing of some of these strains.

Background: Over the last few years, we have observed an increasing number of undiagnosed, but suspected cases of *E. coli* diarrhea in calves. Such observation is not limited to Saskatchewan, but has also been reported in other geographical regions (Hines N. et al. 2001, American Association of Veterinary Laboratory Diagnosticians, 44th annual meeting. Abstract p.33). These observations suggested that there might exist emerging *E. coli* pathotypes causing calf neonatal diarrhea, pathotypes for which we do not routinely test for in the diagnostic laboratory.

Project objectives: our objectives were to identify emerging pathotypes of *E. coli*, if any, and to determine the antibiotic profile of major pathotypes with potential role in diarrhea of calves.

Description of work done: a total of 408 and 635 *E. coli* isolates were recovered from feces and intestines of newborn to 2 week-old diarrheic and non-diarrheic calves in Wetsrn Canada (Saskatchewan, Manitoba, Alberta and British Columbia). The isolates were identified using standard biochemical tests for identification of enterobacteria. Confirmed *E. coli* isolates were tested for the presence of F4, F5, F6, F17 and F41 fimbriae, for *E. coli* attaching and effacing (EAE), porcine attaching and effacing associated (Paa) and for adhesin involved in diffuse adherence (AIDA-I) factors. In addition, the isolates were tested for the heat stable enterotoxins (STa, STb, EAST-1), heat labile enterotoxin (LT), Shiga toxins (Stx1/Stx2), for cytotoxic necrotizing factors (CNF1/CNF2) and for cytolethal dystending toxins (CDT-3/CDT-4) using DNA hybridization. Furthermore, isolates from major pathotypes were tested for sensitivity to 12 antimicrobial drugs such as ampicillin, ampicillin/sulbactam, ceftiofur, enrofloxacin, erythromycin, florfenicol, gentamicin, penicillin/novobiocin, spectinomycin, tetracycline, tilmicosin and trimethoprim-sulfamethoxazole, commonly used in cattle farming. Due to budget constrain, 50 selected isolates from the different pathotypes of interest were O-serotyped.

Observations: Our study provides evidence of existence of distinctive *E. coli* pathotypes referred to as F17/EAST1 and F17/CDT-3/CNF associated or not (±) with EAST1, in calves.

Isolation of strains expressing simultaneously the virulence-associated factors above is of high interest and suggests that related pathotypes may have a potential role in diarrhea of newborn calves. Isolates of pathotypes from diarrheic animals appeared frequently more resistant to antimicrobial drugs tested for than isolates from non-diarrheic animals, suggesting induction of antimicrobial resistance of isolates related to treatment, in the diarrheic group. Development of such resistance may render antibiotic therapy inefficient and be a threat to cattle farming.

Discussion and conclusion: Our data shows that a high number of *E. coli* possess DNA-related sequence for F17 fimbriae in both diarrheic (44.1%) and non-diarrheic (34.3%) newborn to 2-week-old calves. Among these strains, EnteroAggregative heat Stable Enterotoxin 1 (EAST1) Cytolethal Distending Toxin 3 (CLDT-3) and Cytotoxic Necrotizing Factor (CNF) were present simultaneously in a high number of *E. coli* isolates referred to as F17/EAST1 and F17/CDT-3/CNF ± EAST1 pathotypes. F17/EAST1 represented 14.5% and 6.3% of isolates from diarrheic and non-diarrheic calves, respectively, and F17/CDT-3/CNF ± EAST1 represented 5.4% and 10.5%, respectively. The majorities (90%) of these pathotypes were found in newborn to 10 day-old calves of both groups. Other *E. coli* pathotypes such as F17/CDT-3, F17/CNF and CDT-3/CNF were also isolated from both groups of calves and represented approximately 1% of isolates. Although all of the pathotypes above were found in both diarrheic and non-diarrheic calves, it is possible that the non-diarrheic calves were asymptomatic carriers and may develop diarrhea only under certain circumstances. For example, we have reported that the EAE pathotype isolated from non-diarrheic pigs produced diarrhea and show the attachment and effacement of intestinal epithelial cells characteristics of EAE, when inoculated in newborn colostrum deprived pigs (Ngeleka et al. 2003, J. Vet. Diagn. Invest. 15:242-252, ADF funded). These observations suggested that piglets from which the organism was isolated might have developed diarrhea later after specimen collection or may develop diarrhea only in certain circumstances such as under colostrum deprivation. Similarly, an F4/LT/STb pathotype of porcine enterotoxigenic *E. coli* has been isolated from non-diarrheic pigs although strains of this pathotype have been frequently associated with neonatal and post-weaning diarrhea in pigs.

Based on these observations, we believe that although *E. coli* F17/EAST1 and F17/CDT-3/CNF ± EAST1 pathotypes were isolated in high numbers from both diarrheic and non-diarrheic calves, it is necessary to determine their role, if any, in neonatal diarrhea of calves. Few studies have reported the presence of F17, EAST1, CDT-3 and CNF factors in both diarrheic and non-

diarrheic calves [Osek, J. 2001. J. Vet. Med. 48 (9):641-646; Mainil, J. 1997. Can. J. Vet. Res. 61 (3):193-199]. These reports suggested that the virulence-associated factors above might be considered potential virulence determinant of *E. coli* causing diarrhea or septicemia, or both, in calves. However, the role of these factors in neonatal diarrhea has not been well established. Our study agrees with such reports and moreover provides evidence that these factors can be expressed simultaneously in one *E. coli* strain, referred to as F17/EAST1, F17/CDT-3/CNF ± EAST1 pathotypes. In addition, our study provides an opportunity to determine and document the role of such pathotypes in production of diarrhea in experimentally inoculated newborn calves.

EAST1 enterotoxin has been associated with diarrhea caused by Enteroaggregative *E. coli* (EAggEC). However, we have demonstrated recently that EAST1 alone does not seem to play a role in diarrhea of pigs (J. Vet. Diagn. Invest. 15:242-252, 2003). Nevertheless, we feel it is still necessary to determine and document whether or not this enterotoxin, as associated with F17 fimbriae, may play a role in calves' diarrhea and subsequently help establish its host-related specificity, if any.

Enterotoxigenic *E. coli* pathotype F5/STa/Paa was isolated only from diarrheic calves (4.4% of isolates, table 1). The role of F5 (K99) fimbriae and STa enterotoxin in such pathotype is well known. However, it was interesting to find that Paa factor was always associated with this pathotype, as well as the EAE pathotype. Paa DNA sequence has been reported in *E. coli* isolated from pigs, and has been associated with the attaching and effacing activity of EAE in vitro. Our study provides evidence that Paa is not limited to pigs, but is also distributed in *E. coli* isolates from calves. Presence of this factor in some pathotypes may increase their pathogenicity.

In conclusion, we have demonstrated the existence of emerging *E. coli* pathotypes in Western Canada. Virulence-associated factors associated with these pathotypes may be considered significant, in development of diarrhea in newborn calves. It is clear that despite the implementation of good vaccination program against neonatal calf diarrhea using current ETEC anti fimbrial-based vaccines, calves infected with such pathotypes may not be protected. In addition, the increase of resistant strains in the diarrheic group will render antibiotic therapy inefficient.

Further development: we hypothesis that F17 fimbriae, EAST1, CDT-3 and CNF are considered potential virulence-associated factors for *E. coli* causing diarrhea or septicemia, or both, in calves. Our discovery of simultaneous expression of such factors in the F17/EAST1 and F17/CDT-3/CNF ± EAST1 pathotypes would increase the virulence and subsequent enteropathogenicity of such strains in calves. This can be documented through in vivo experimental infection of newborn calves and has been part of a future project proposal, which will nicely complement the present report.

(d) PERSONNEL

Mrs. Jane Moker was hired as a technician and had worked part time on the project from November 2002 to May 2003.

(e) No major equipment was purchased with project funds.

(f) The results of the project will be presented at the Western Canadian Association of Bovine Practitioners annual meeting and the Canadian Animal Health Network annual meeting (2004).

(g) No photos were taken.

(h): Saskatchewan Agricultural development Fund and the Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan names are mentioned in the Acknowledgment section of all presentations and publications.

Table 1. Occurrence of the major *E. coli* pathotypes among diarrheic and non-diarrheic calves.

		E. coli isolates	
Origin	Number tested	Major pathotype*	Number Positive (%)
Diarrheic calves	408	EAST1	100 (24.5)
		F17	82 (20.1)
		F17/EAST1	59 (14.5)
		F17/CDT-3/CNF ± EAST1	22 (5.4)
		F5/STa/Paa ± (F17, F41, EAST1)	18 (4.4)
		EAE/Paa/EAST1 ± Stx1	10 (2.5)
No diarrheic calves	635	EAST1	96 (15.1)
		F17	90 (14.2)
		F17/EAST1	40 (6.3)
		F17/CDT-3/CNF ± EAST1	67 (10.5)
		EAE/Paa/EAST1 ± (CDT-3, CNF, Stx1, Stx2)	27 (4.2)

* Minor pathotypes, accounting for approximately 1 % each, included F17/CDT-3, F17/CNF, CDT-3/CNF, AIDA-I, AIDA-I/EAST1, AIDA-I/CDT-3/EAST1 and a variety of combinations between F17, STa, CDT-3, EAE, Paa, Stx1 and Stx2 from both diarrheic and non-diarrheic animals.

Table 2. Antibiotic resistance profile of the main *E. coli* pathotypes recovered from diarrheic and non-diarrheic calves.

		E. coli isolates							
Origin/ Pathotype	Total number	Percentage resistant to antibiotic[a]							
		AMP	AMP/S	XLN	ENO	FFC	GM	ADS	SXT
Diarrheic									
F17/EAST1	59	52.5	40.7	3.4	0.0	23.7	1.7	25.4	67.8
F17/CDT-3/CNF[b]	22	31.8	9.0	0.0	0.0	13.6	4.5	9.0	36.4
F5/STa/Paa[c]	18	66.6	44.4	5.5	0.0	11.1	0.0	61.1	33.3
EAE/Paa/EAST1[c]	10	40.0	40.0	10.0	0.0	20.0	0.0	30.0	40.0
Non-diarrheic									
F17/EAST1	40	12.5	7.5	0.0	0.0	2.5	2.5	15.0	17.5
F17/CDT-3/CNF	67	14.9	7.5	0.0	0.0	1.5	3.0	13.4	13.4
EAE/Paa/EAST1[c]	27	11.1	7.4	0.0	0.0	0.0	0.0	7.4	7.4
Total diarrheic	109	67.9	34.8	3.6	0.0	19.2	1.8	28.4	53.2
Total non-diarrheic	134	13.4	7.5	0.0	0.0	1.5	2.2	12.7	13.4

[a]: AMP (ampicilinn, 10 μg), SAM (ampicillin-sulbactam, 20 μg), XNL (ceftiofur, 30μg), ENO (enrofloxacin, 5 μg), FFC (florphenicol, 30 μg), GM (gentamicin, 10μg), ADS (spectinomycin, 100 μg), SXT (sulfamethoxazole- trimethoprim 23.75/1.25 μg).

[b]: pathotype associated or not with EAST1

c: pathotypes associated or not with F17, F41, EAST1, CDT-3, CNF, Stx1, or Stx2